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PRINCIPAL INVESTIGATOR: Lawrence F. Povirk, Ph.D.

CONTRACTING ORGANIZATION: Virginia Commonwealth University  
Richmond, Virginia 23298

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**6. AUTHOR(S)**

Lawrence F. Povirk, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**Virginia Commonwealth University  
Richmond, Virginia 23298

E-Mail: LPOVIRK@hsc.vcu.edu

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To examine the possible role of the breast cancer susceptibility factor BRCA1 in the nonhomologous end joining pathway of DNA double-strand break repair, an assay to detect both accurate and inaccurate end joining events in intact cells was devised. A fusion gene of green fluorescent protein (EGFP) and neomycin resistance (*neo*) was constructed with a linker containing cleavage sites for the enzymes BcgI and I-SceI. BcgI induces two simultaneous breaks, excising a 34-bp segment of DNA. Accurate repair of such a break would be detected as a 34-bp deletion that would put *neo* back in-frame and confer G418 resistance. Inaccurate repair events of both BcgI- and I-SceI-induced breaks would similarly be detected. A lentivirus vector was constructed to introduce the fusion gene into human cells, and transductants of MCF-7 breast tumor cells were isolated. Although development of the assay system is not yet complete, proof-of-principle experiments showed that deletions could indeed be generated by I-SceI-induced cleavage and detected as reactivation of the *neo* gene. Intracellular DNA cleavage by BcgI, introduced into cells by electroporation was also demonstrated, although it remains to be determined whether such cleavage will be sufficient to detect repair and assess repair accuracy.

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## Introduction

Mutations in the breast cancer susceptibility gene *BRCA1* are responsible for most cases of hereditary breast cancer [1]. *BRCA1*-deficient cells derived from such hereditary tumors show reduced double-strand break repair by the homologous recombination repair pathway [2]. The possible role of *BRCA1* in the more common nonhomologous end joining (NHEJ) pathway has been controversial, with reports of either deficiency or normal repair in *BRCA1*-deficient mouse or human cells, or extracts thereof [3-5]. One possible explanation of these disparate data is that *BRCA1* affects the accuracy but not the efficiency of NHEJ. This hypothesis, as well as other hypotheses regarding the determinants of NHEJ accuracy, have been difficult to test because most *in vivo* end joining assays detect repair by virtue of the mutations that often occur during end joining of enzymatically induced site-specific DSBs [6,7]; thus, simple, accurate religations of such breaks are normally invisible to these assays. The intent of work funded by this Concept Award was to develop an *in vivo* assay that would detect both accurate and inaccurate end joining events, so that effects of deficiencies in *BRCA1* and other repair factors could be more easily assessed. The proposed assay was based on cleavage with the unusual restriction enzyme BcgI, which excises a 34-bp DNA segment by making two adjacent simultaneous DSBs [8]. Thus, accurate rejoining of the resulting ends should produce a 34-bp deletion, which could be detected by activation of a neomycin-resistance (*neo*) marker fused to an enhanced green fluorescent protein (EGFP) gene through a cleavable linker (*neo* expression confers neomycin resistance in bacterial cells and G418 resistance in mammalian cells).

## Task 1: Construct GFP-linker-*neo* fusion gene

For construction of a vector with the enhanced green fluorescent protein (EGFP) gene fused to the neomycin-resistance gene through a linker containing target sites for enzymatically induced DSBs, the starting materials were the plasmids pEGFP-1 and pIRESneo3, purchased from Clontech (Fig. 1). First, the EGFP coding sequence was isolated from pEGFP-1 as a NotI-BamHI fragment and ligated into the multicloning site in pIRESneo3. To fuse the EGFP and *neo* genes, the resulting vector was cut at the 3' end of EGFP with BsrGI, and just upstream of the 5' end of *neo* with XmaI. A 96-bp linker with 5' overhangs (CCGG for XmaI; GTAC for BsrGI) was purchased from Integrated DNA Technologies

and ligated into the cleaved vector (Fig. 2). The resulting construct pEGPflinkerneo allows translation of the full EGFP reading frame driven by a cytomegalovirus (CMV) promoter, immediately followed by two termination codons in the linker (Fig. 2A). The *neo* gene begins just downstream from the linker, but is out-of-frame with respect to EGFP and has no ribosomal entry site of its own; hence, no *neo* expression from the initial construct is expected.

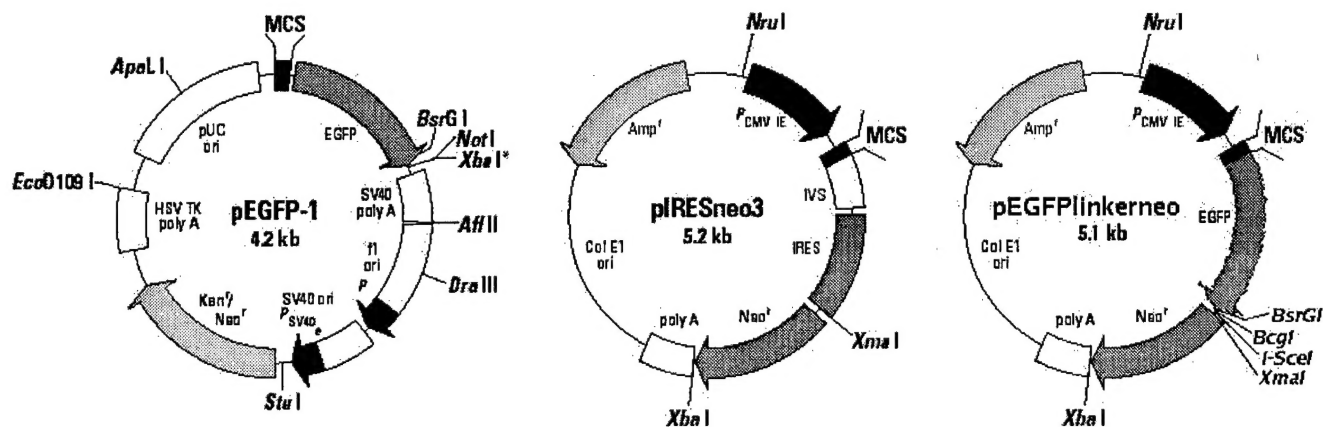


Figure 1. The starting plasmids pEGFP-1 and pIRESneo3 from Clontech, and the constructed DSB repair substrate plasmid pEGPflinkerneo. MCS = multicloning site, CMV = cytomegalovirus.

The linker was designed with two restriction sites, a BcgI site and an I-SceI site (Fig. 2A). The principle of the proposed double-strand break repair assay is that cleavage with BcgI, which cuts at two sites and excises a 34-bp segment of DNA, would leave complementary -GT and -AC 3' overhangs. Accurate joining of the resulting ends would shift the termination codons out-of-frame while shifting *neo* back in-frame, to generate an EGFP-*neo* fusion gene and protein (Fig. 2B) that should render cells G418-resistant [9]. As outlined in the initial proposal, the BcgI site is flanked on each side by an ATCC microhomology (bolded letters in Fig. 1); repair by exonucleolytic resection of the BcgI-generated ends followed by annealing of the ATCC microhomology would also result in an active EGFP-*neo* fusion protein, but with a slightly shorter linker (Fig. 2C).

To increase the flexibility of the vector as a DSB repair substrate, an I-SceI site (18-bp recognition sequence) was also incorporated into the linker. Accurate joining of an I-SceI-induced break would not change the sequence, but resection-based joining could give an active EGFP-*neo* fusion gene and protein whenever  $3n+1$  bp are deleted. Although I-SceI thus has the disadvantage that accurate joining events are not detected, it has two distinct advantages over BcgI; first, there are few if any other I-SceI sites in any mammalian genome (assuring that a high frequency of site-specific breaks in the linker could be induced without significant cytotoxicity), and second, I-SceI can be efficiently expressed in a variety of human cell types by transduction with an existing adenoviral vector [10].

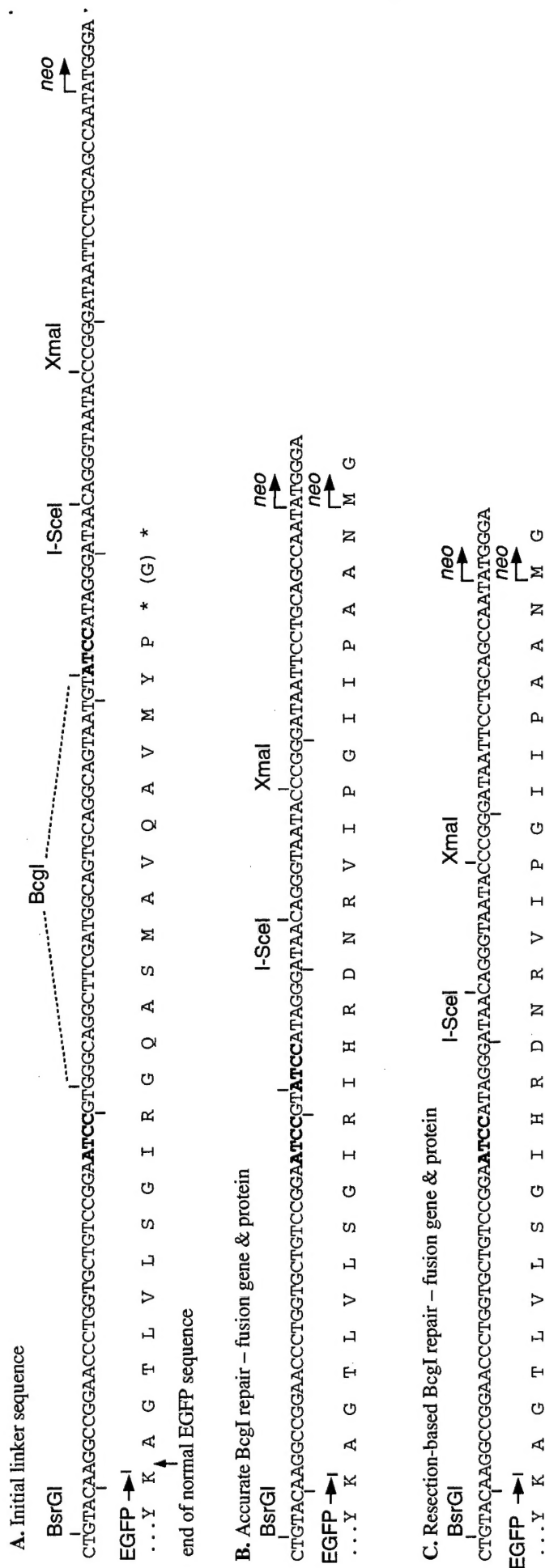


Figure 2. The initial DSB repair substrate generated by insertion of a synthetic 96-bp BsrGI/XmaI linker between the EGFP and *neo* genes (A), and the products generated by accurate end joining (B) and resection-based end joining at an ATCC (bold letters) microhomology (C). Lower line in each panel shows the predicted amino acid sequence (\* = termination codon).

## Task 2. Create adeno-associated viral vector

In the original research plan, we proposed to introduce the EGFP-linker-*neo* construct into various cell lines using an adeno-associated viral (AAV) vector. However, recent literature and our own recent experience with other transgenes in the AAV system have revealed several unanticipated complications. Investigations into the details of AAV DNA metabolism [11,12] have indicated that virus with duplicated genomes are often produced, and that under some conditions host sequences can be rearranged upon integration and random AAV integration can occur (thus negating one of the main putative advantages of AAV). Most disturbingly, when we prepared an AAV vector containing a mutant EGFP that could form an active, wild-type gene by homologous recombination following cleavage at an I-SceI site in the vector, we did not obtain the expected transductants that specifically expressed EGFP following infection with I-SceI-expressing virus, as we had seen when the same mutant EGFP gene was chemically transfected into cells [10]. Instead, a few AAV transductants constitutively expressed EGFP, while the majority of transductants did not express EGFP and did not yield any EGFP-expressing clones following adenovirus-mediated I-SceI expression. These results suggest that either the AAV vector was rearranged upon integration into the host, or that there were other anomalous effects that either prevented homologous recombination or blocked expression of the resulting wild-type EGFP allele.

We therefore chose instead to construct a lentivirus containing the EGFP-linker-*neo* cassette. This virus could then be used to introduce the cassette into virtually any human cell, albeit not site-specifically. For this purpose the entire EGFP-linker-*neo* cassette (without CMV promoter) was isolated from pEGFPlinkerneo as a BamHI / XbaI fragment. An SpeI linker was added to the XbaI end and the fragment was ligated between the BamHI and SpeI sites of the lentiviral vector pWPXLd [13]. The resulting replication-defective vector (Fig. 3) contains the EGFP-linker-*neo* cassette, driven from the elongation factor 1 (EF1) promoter. Transfection of this vector into 293T packaging cells produces infectious but replication-defective lentivirus containing the EGFP-linker-*neo* cassette (as well as other sequences between the long terminal repeats (LTRs)). Transduction of any human cell line with this virus should result in integration of the cassette into the cellular genome.

## Task 3. Create and characterize GFP-linker-*neo* integrant cell line

While construction of the EGFP-linker-*neo* lentivirus was in progress, several attempts were made to generate stable cell lines by transfection of the pEGFPlinkerneo plasmid. Transfection of *BRCA1*-deficient HCC1937 human colon cancer cells, using Superfect transfection reagent (Invitrogen) failed to yield any stable green-fluorescent cells. Transfection of Chinese hamster ovary cells (chosen because they are normally have a relatively high transfection efficiency) yielded several clones that expressed green fluorescence but were still G418-sensitive (suggesting that *neo* was not expressed, as expected). However, upon continued culture, each of 4 lines lost green fluorescence, suggesting that the EGFP-linker-*neo* construct was not stably integrated in these clones. We therefore constructed the lentivirus vector pWPXLd-EGFPlinkerneo (Fig. 3), which should give much more efficient and reliable stable integration of the EGFPlinkerneo cassette into any human cell line. In a preliminary experiment, MCF-7 breast tumor cells and *Brc1*-deficient HCC1937 cells were transduced with packaged virus containing the EGFPlinkerneo cassette. Although no integrant clones of HCC1937 cells have yet been observed, several stable, weakly fluorescent MCF-7 clones have been isolated and are currently being characterized.

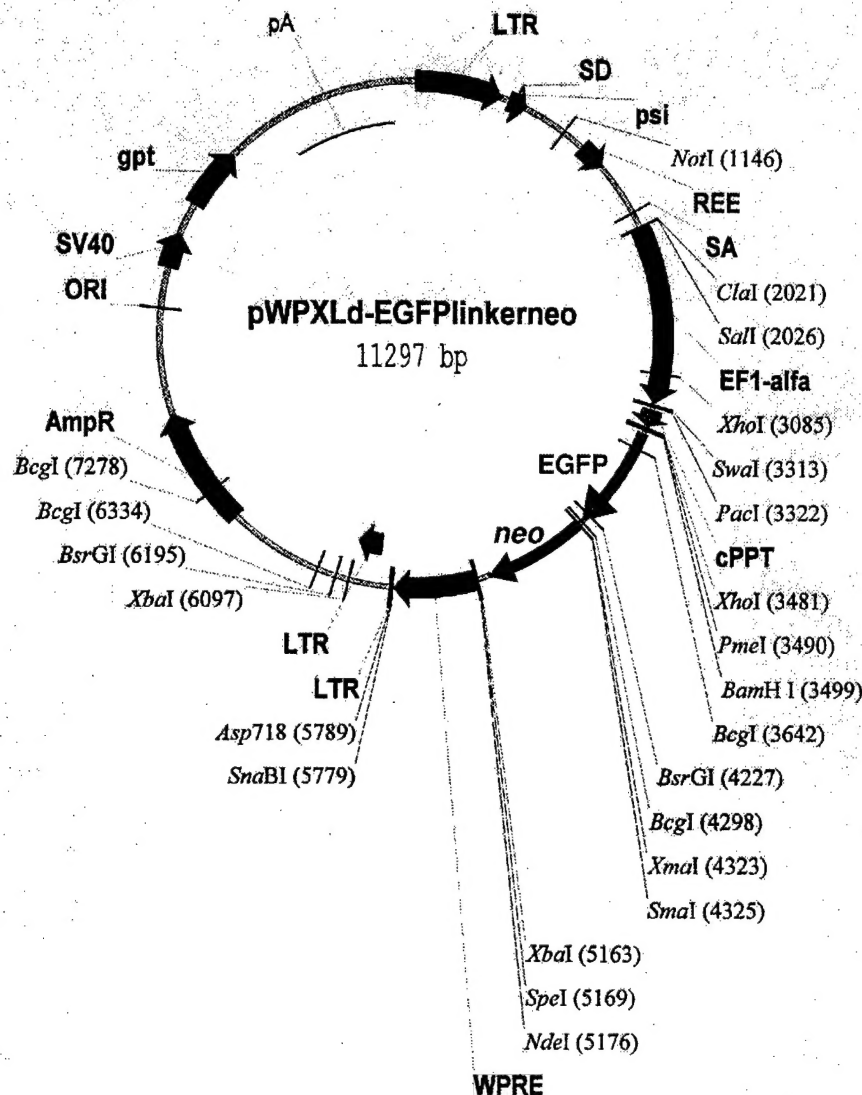


Figure 3. A lentivirus vector for introduction of the EGFPlinkerneo DSB repair substrate into human cells.

#### Task 4. Treat integrant cell line with BcgI and characterize G418-resistant clones

When it became apparent that considerable additional work would be required to obtain stable GFP-linker-*neo* cell lines, a proof-of-principle experiment was carried out in which CHO cells were transiently transfected with pEGFPlinkerneo, either with or without the I-SceI-expressing vector pCMV-I-SceI, and as a control, with pIRESneo3 (see Fig. 1). Transfection of pEGFPlinkerneo alone yielded some G418-resistant (*i.e.*, *neo*-expressing) clones, but the frequency was higher when pCMV-I-SceI was also transfected. Most of the G418-resistant clones from the pEGFPlinkerneo transfectants showed weak green fluorescence, suggesting stable expression of both EGFP and *neo*. Genomic DNA



was isolated from one clone from each transfection condition and subjected to PCR with primers flanking the linker. In the case of cells transfected with pEGFPlinkerneo plus pCMV-I-SceI, three PCR products were generated, including a doublet at the predicted size of the unaltered linker (361 bp), and fragment of ~280 bp (Fig. 4). Upon longer electrophoresis (inset), the doublet was resolved to two distinct bands, and all three bands were isolated from the gel and subjected to DNA sequencing. The longest fragment showed the original, unaltered sequence. The ~280-bp fragment gave no readable sequence, but the shorter fragment of the doublet showed a 22-bp deletion at the I-SceI site, which would restore the *neo* reading frame (Fig. 5). This result suggests that DSB repair can indeed generate, from the EGFP-linker-*neo* construct, a fusion protein with both EGFP and *neo* activity.

Although we have not yet generated stable EGFP-linker-*neo* transfectants, we sought to determine whether electroporation with BcgI could in fact induce significant DNA cleavage in intact cells. Such intracellular DNA cleavage would be essential for the DSB repair assay, and cleavage has been demonstrated with several other electroporated restriction enzymes [14]. Initial experiments with MCF-7 breast tumor cells (data not shown) indicated that electroporated BcgI was less toxic to cells than would have been expected based on studies with other restriction enzymes. We therefore initiated experiments with Chinese hamster ovary cells (which produce results much faster due to their faster growth rate) in order to define conditions for successful BcgI electroporation. Electroporation alone at 400 volts

with a 250  $\mu$ F capacitor but without any restriction enzyme reduced cell survival approximately twofold (Table I). Addition of 125 units/ml of BcgI to the electroporation buffer consistently reduced survival by an additional ~40-50%. Although BcgI requires *S*-adenosylmethionine (SAM) for optimal activity (an unusual feature for a restriction enzyme), endogenous cellular levels of SAM appeared to be sufficient for activity as addition of SAM to the electroporation medium did not increase the cytotoxicity of electroporated BcgI. Electroporation of BcgI at 450 volts gave less consistent results, perhaps because of the greater cytotoxicity of electroporation alone, which at this voltage reduced survival approximately fivefold (Table I). A more typical restriction enzyme, HaeIII, was much more cytotoxic than an equal quantity of BcgI, perhaps because the complex mechanism of BcgI requires that a longer segment of DNA be accessible to the enzyme.

To more directly assess whether electroporated BcgI induced DSBs in the cells, the cells were plated at 150,000 per 25-cm<sup>2</sup> flask, incubated for two days, and then stained with Giemsa. Micronuclei were then scored, in double-blinded fashion. Micronuclei are formed when unrepaired DSBs give rise to chromosome fragments that fail to properly segregate at mitosis [15]. Electroporation alone at 400 volts,

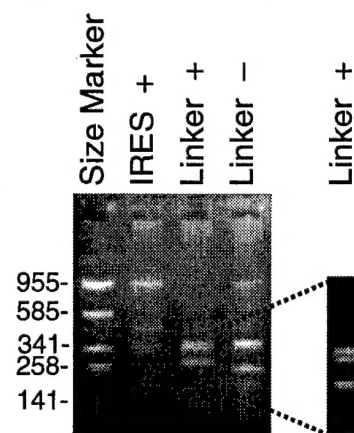


Figure 4. PCR of DNA from cells transfected with pIRESneo3 (IRES) or pEGFPlinkerneo (Linker), either with (+) or without (-) the pCMV-I-SceI. Expanded inset shows Linker + sample run for a longer time.

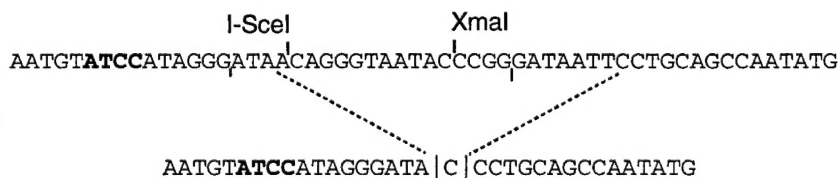


Figure 5. Deletion resulting from I-SceI-induced DNA cleavage, with a net deletion of 22 bp. Dashed lines show the segment that was deleted. Vertical lines denote sequences derived from each side of the break, with apparent insertion of a single C at the breakpoint.



even though it was cytotoxic, produced very few micronuclei. BcgI electroporation, however, increased the incidence of micronuclei approximately tenfold, suggesting that it did induce DSBs in intact cells. As in the survival assays, HaeIII was much more effective than BcgI, with nearly half of the cells being micronucleated, a 100-fold increase over electroporation alone.

**Table I. Cell killing and induction of micronuclei by electroporated BcgI.** CHO-K1 cells ( $10^6$ /ml) were electroporated at the indicated voltage using a Bio-Rad Gene Pulser with a 250  $\mu$ F capacitor, in the presence of BcgI, HaeIII, or BcgI storage buffer alone ("None"). In some cases S-adenosylmethionine (SAM, 8  $\mu$ M) was added. One aliquot was then plated at 800 cells per dish and colonies counted 8 days later. Another aliquot was plated at 150,000 cells/flask and two days later micronucleated (MN) cells were scored.

Enzyme	Voltage	SAM	Colonies	MN/total	%MN
None	0	-	286	1/650	0.15
None	400	-	147	2/558	0.36
BcgI	400	-	94	20/550	3.6
None	400	+	192	3/519	0.58
BcgI	400	+	115	29/541	5.4
None	450	-	36	5/534	0.9
BcgI	450	-	17	5/511	1
None	450	+	39	25/540	4.6
BcgI	450	+	61	30/531	5.6
HaeIII	400	-	17	103/233	44.2

## Key Research Accomplishments

- Generation of fusion gene of EGFP and *neo*, with cleavable linker
- Proof-of-principle demonstration that DSB repair can be detected with the fusion gene
- Demonstration of intracellular DNA damage following electroporation of BcgI

## Reportable Outcomes

Because the project was directed at technical assay development, there have been as yet no publications resulting from the work. The main outcomes of the project thus far have been the vectors generated, as follows.

- Construction of vector pEGFPlinkerneo with EGFP fused to *neo* through a cleavable BcgI / I-SceI linker.
- Construction of a lentiviral vector pWPXLd-EGFPlinkerneo as well as infections lentivirus stock containing the EGFP-linker-*neo* cassette.
- Isolation of MCF-7 breast tumor cells carrying the EGFP-linker-*neo* cassette, from lentivirus-infected cells

## Conclusions

In the execution of the project, several unanticipated technical difficulties were revealed, including difficulty in obtaining transfectants of HCC1937 cells, anomalous behavior of the AAV vectors, and apparent inefficiency of electroporated BcgI in cleaving DNA in intact cells. Nevertheless, the principle of the assay was verified, namely, that a cryptic *neo* gene could be activated by DSB repair-mediated in-frame fusion to an EGFP gene directly upstream. Despite termination of CDMRP funding, we are continuing with development of the assay, using primarily institutional funds. We expect that within the next year, we will be able to assess the specificity of repair of at least I-SceI-induced DSBs in a variety of cell lines using the lentivirus vector to integrate the DSB target sequence, and then an adenoviral vector to express I-SceI. Further experiments will be required to determine whether enough site-specific DSBs will be generated by electroporated BcgI, at reasonable levels of cell survival, to permit assessment of repair accuracy, as originally proposed. It is possible that expression of BcgI from an adenovirus vector, which in theory should be relatively simple to construct, may yield better results than electroporation.

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**Personnel receiving support from this award:**

Lawrence F. Povirk, Professor of Pharmacology and Toxicology  
Kristoffer Valerie, Professor of Radiation Oncology  
Tong Zhou, Postdoctoral Employee